CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors:Enrico Bardone, Marco Bravi,Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN**2283-9216
DOI: 10.3303/CET1438055

Miniaturized Culture for Heterotrophic Microalgae Using Low Cost Carbon Sources as a Tool to Isolate Fast and Economical Strains

Annamaria D. S. Vidotti, Renato S. Coelho, Liliana M. Franco, Telma T. Franco*

State University of Campinas - School of Chemical Engineering -Av. Albert Einstein 500, 13083-852, Campinas-SP, Brazil
*franco.feq@gmail.com

Microalgae are well known for their ability to grow photoautotrophically, however higher biomass yields have been reported when microalgae was grown heterotrophically. The feasibility of large scale cultures of microalgae in heterotrophic conditions is still limited by, among other things, the high cost of nutrients and organic substrates used in this type of cultivation. This work aims to explore the utilization of different low cost carbon sources for the cultivation of two different strains of microalgae. Cassava wastewater, sugarcane molasses, glycerol, xylose, sucrose and sodium acetate were tested as carbon sources for *Chlorella vulgaris* and *Scenedesmus bijugus* cultures. Glucose and fructose were also tested as reference carbon sources. The methodology of miniaturized growth allowed the screening of different substrates and conditions of the medium much faster than other methodologies (shaking flasks, bioreactor). The results demonstrate the feasibility of the miniaturized culture methodology in the development and evaluation of heterotrophic cultivation of microalgae. The main observed problems were settling of cells, evaporation of the medium during the experiment and interference in spectrophotometric reading (caused by water condensation on the lid), which had to be solved to adapt the methodology of miniature culture to heterotrophic microalgae cultivation. The evaluated microalgae strains presented different growth behaviours in the different carbon sources tested. Cassava wastewater and sugarcane molasses hydrolyzed allowed higher biomass production and proved to be a suitable low cost substrate for increasing algae-based processes feasibility.

1. Introduction

Microalgae enables numerous uses for biotechnological industries, they can produce products with high-value like pigments, carbohydrates, lipids, proteins and vitamins, that have application in feed supplement, cosmetic, health food, chemical and pharmaceutical industries (Suali and Sarbatly, 2012). In addition these photosynthetic microorganisms can be applied in the bioenergy industry (Oncel, 2013; Mata et al. 2010).

Microalgae are photosynthetic organisms naturally, therefore the most common process for the cultivation of microalgae is the autotrophic growth, since many are efficient converters of solar energy (Richmond, 2004). However heterotrophic microalgal metabolic pathways show significant productivity gains in biomass when compared with the conventional photosynthetic systems (Perez-Garcia et al. 2010).

In heterotrophic cultivation organic carbon sources are used, such as sugars or organic acids, as a source of carbon and energy eliminating the requirement for light, and in the literature was reported that various algal species can grow in the dark, assimilating a wide variety of organic carbon sources (Malcata, 2011; Mata et al. 2010). But among other problems of this type of culture, the cost of the carbon source is one of the most discussed drawbacks that can hinder the commercial application of that pathway (Suali and Sarbatly, 2012). An alternative is replacement of carbon sources and major synthetic nutrient medium for growing heterotrophic organic residues (Liang, 2013; Perez-Garcia et al. 2011).

The agroindustrial sector generates a considerable amount of residues rich in sugars, acids, nitrogen and other nutrients (Liang, 2013). At the same time, the microalgae can be used to reduce the load of organic and inorganic industrial effluents (Mitra et al. 2012; Wu et al. 2012). However some wastewater may be

Please cite this article as: Vidotti A., Coelho R., Franco L.M., Franco T.T., 2014, Miniaturized culture for heterotrophic microalgae using low cost carbon sources as a tool to isolate fast and economical strains, Chemical Engineering Transactions, 38, 325-330
DOI: 10.3303/CET1438055

toxic to support microalgal growth (Liang, 2013), because this it is necessary to screen microalgal species that can grow in this type of substrate.

With the methodology of miniaturized growth high number of parallel experiments that can be carried, furthermore the use microwell plates allowed the screening of different algal species, substrates, nutrients and conditions of the medium much faster than other methodologies (shaking flasks, bioreactor). This small-scale systems is used for a first screening and should be scalable to the pilot (Hiliig et al. 2014).

This work aims to explore the utilization of different low cost carbon sources for the cultivation of two different strains of microalgae. Cassava wastewater, sugarcane molasses, glycerol, xylose, sucrose and sodium acetate were tested as carbon sources for *Chlorella vulgaris* and *Scenedesmus bijugus* cultures. Glucose and fructose were also tested as reference carbon sources. In addition this paper will analyze the feasibility of the miniaturized culture methodology in the development and evaluation of heterotrophic cultivation of microalgae.

2. Materials and methods

2.1 Microalgae and medium

Chlorella vulgaris (CPCC 90) was from the Canadian Phycological Culture Centre and *Scenedesmus bijugus* was from the Culture Collection of Freshwater Microalgae, Department of Botany, Federal University of São Carlos (WDCM: UFSCar CC 835). Stock cultures were maintained axenically on synthetic modified BBM medium (Stein-Taylor, 1973) with glucose (10 $g.L^{-1}$ for *C. vulgaris* and 5 $g.L^{-1}$ for *S. bijugus*).

Composition of the synthetic modified BBM medium ($mg.L^{-1}$): Na_2EDTA (50), KOH (3,1), $CaCl_2.2H_2O$ (25), $MgSO_4.7H_2O$ (75), K_2HPO_4 (75), KH_2PO_4 (175), NaCl (25), MoO_3 (0,71), $Fe_2SO_4.7H_2O$ (4,98), H_2SO_4 (1 $\mu L.L^{-1}$), H_3BO_3 (11,42), $ZnSO_4.7H_2O$ (8,82), $MnCl_2.4H_2O$ (1,44), $CuSO_4.5H_2O$ (1,57), $Co(NO_3)$ $2.6H_2O$ (0,49), $NaNO_3$ was added as function of carbon source to achieve the C/N ratio of 20, pH 6.8.

2.2 Carbon source

The medium was supplemented with different carbon sources, in order to grow *C. vulgaris* and *S. bijugus* respectively: glucose (10 and 5 $g.L^{-1}$), sodium acetate (10 and 5 $g.L^{-1}$), hydrolyzed sucrose (20 and 12 $g.L^{-1}$), hydrolyzed sugarcane molasses (30 and 15 $g.L^{-1}$), fructose (2 and 5 $g.L^{-1}$) and cassava wastewater supplemented with BBM medium salts. The concentrations of hydrolyzed sucrose, hydrolyzed sugarcane molasses and cassava wastewater were based for 10 $g.L^{-1}$ of glucose. The glycerol, sucrose and xylose were tested at 2 $g.L^{-1}$ for the two strains.

Hydrolyzed sucrose was achieved by mixing sucrose solution (500 $g.L^{-1}$) corresponding to 0.0037 % (w/v) and 0.0183 L HCl 3M; hydrolyzed sugarcane molasses were prepared by mixing 0.1372 L of HCl 3M with sugarcane molasses solution (500 $g.L^{-1}$), corresponding to 0.0273% (w/v) (Gao et al. 2010). The solutions were incubated in a water bath at 80 °C for 30 minutes with subsequent cooling in an ice bath to room temperature. The pH was neutralized using NaOH solution 3M. The average yield of the hydrolysis of sucrose was 87.3 ± 4.6 % and of sugarcane molasses was 89.9 ± 0.12 %.

The molasses were detoxified with granular activated carbon in a concentration of 5 % (w/v) with further constant stirring at 200 rpm for 2 hours. After this time the solution was vacuum filtered with filter paper (Whatman No. 1), followed by centrifugation at 10000 rpm at 5 °C for 15 min (Valduga et al. 2007).

2.3 Culture conditions

The cultivations were performed in 48-well plates (with 2 ml well). The culture volume was 0.5 ml. All cultures were incubated in dark, at a rotary rate of 250 rpm, at 26 ºC on orbital shakers. A model VICTOR plate reader X4 ™ (Perkin Elmer) was used in this study to assess cell growth in microplates. The cultures were inoculated with an initial biomass concentration of 0.3 $g.L^{-1}$ for *C. vulgaris* and 0.6 $g.L^{-1}$ for *S. bijugus*. The experiments were in duplicate and our results are expressed as the average of the measured values of the two cultures.

2.4 Biomass

The biomass concentration ($g.L^{-1}$) was obtained by means of the absorbance of the cell suspension at 680 nm, through of the previously established calibration curve. For construction of calibration curve, biomass dry cell weight was measured by filtering a 5 mL sample of the culture broth through a 0.22 µm porosity membrane and drying of the filter in an oven at 50 °C until constant weight.

The maximum specific growth rate ($\mu_{máx}$) was obtained through exponential adjustment in the logarithmic phase of growth.

3. Results

Axenic cultures of *C. vulgaris* and *S. bijugus* were grown in culture media containing different carbon sources: cassava wastewater, hydrolyzed sugarcane molasses, glycerol, hydrolyzed sucrose, xylose and sodium acetate. Glucose and fructose were also tested as reference carbon sources.

3.1 *Scenedesmus bijugus* cultivation

Figure 1 shows the increase in biomass of *S. bijugus* in microplates for different carbon sources tested.

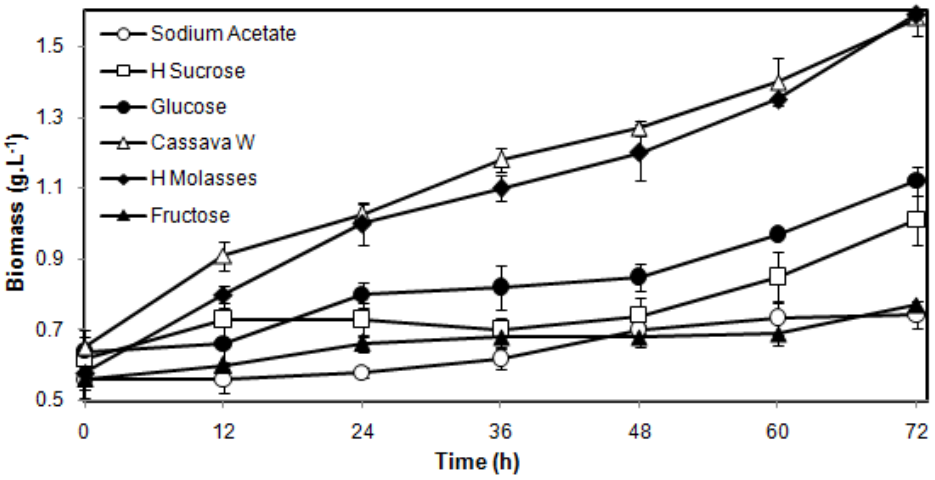

Figure 1: *Growth of S. bijugus in different substrates in micro-plates. 'O' Sodium acetate 5 g.L^{-1}; '□' hydrolyzed sucrose 12 g.L^{-1} (5.0 ± 0.3 g.L^{-1} of glucose); '●' glucose 5 g.L^{-1}; '∆' cassava wastewater (5.0 ± 0.1 g.L^{-1} of glucose); '♦' hydrolyzed molasses 20 g.L^{-1} (5.0 ± 0.1 g.L^{-1} of glucose) and '▲' fructose 5 g.L^{-1}.*

Figure 1 shows that *S. bijugus* reached about 1 g.L^{-1} of biomass in 72 hours using glucose and cassava wastewater as carbon sources. Glucose is one of the most common sources of carbon and the substrate most used for heterotrophic cultivation of microalgae (Suali and Sarbatly, 2012), mainly because the use of glucose allows higher growth rates when compared with other substrates (Perez-Garcia et al. 2010).
However, glucose is a carbon source of high cost (Canakcı and Sanli, 2008). We aimed to study alternative carbon sources of lower cost, which generate similar or higher yields than that observed when glucose was used as carbon source.
Some studies have reported hydrolyzed organic carbon sources such as corn powder hydrolysate (Xu et al. 2006) and sweet sorghum (Gao et al. 2010). The use of hydrolysates in heterotrophic cultivation, in economic terms, can reduce up to 50 % of production costs (Gao et al. 2010; Huang et al. 2011; Yan et al. 2011).
In this sense, hydrolyzed sucrose substrate, which can be obtained mainly from sugarcane, also was tested. The hydrolyzed sucrose provided a slightly less cell growth than glucose in the same culture time (Figure 1).The two substrates that stood out in the experiments were cassava wastewater and hydrolyzed sugarcane molasses (Figure 1). Sugarcane molasses is a byproduct of the sugar industry (Satyawali and Balakrishnan, 2008), and the cassava wastewater is the liquid effluent obtained in the processing of cassava starch and flour, which has high pollution potential (Suman, 2011). Sugarcane molasses and cassava wastewater are mainly composed by sucrose, glucose and fructose and this strain proved capable of consuming fructose (Figure 1). The higher cellular growth associated with these two substrates can be explained by the fact that *S. bijugus* have used glucose and fructose for growth.
Another explanation is that these two substrates not only contain essential macro-nutrients for microorganisms, but can also contain vitamins, metal ions and nitrogen compounds, increasing the growth of microalgae (Huang et al. 2011).
Sodium acetate was also investigated since it is a readily available and inexpensive substrate to be derived from a number of industrial applications. It is also a fairly common source of carbon for many microbial species (Bumbak et al. 2011). With sodium acetate *S. bijugus* showed a much lower growth compared to other substrates (Figure 1).

3.2 *Chlorella vulgaris* cultivation

The same substrates were analyzed in the minutiarized growth of *C. vulgaris*, and the results are show by Figure 2 (now with the strain of *C. vulgaris*). From Figure 2 it is observed that in general, higher cell concentrations are achieved with *C. vulgaris* when compared with *S. bijugus*.

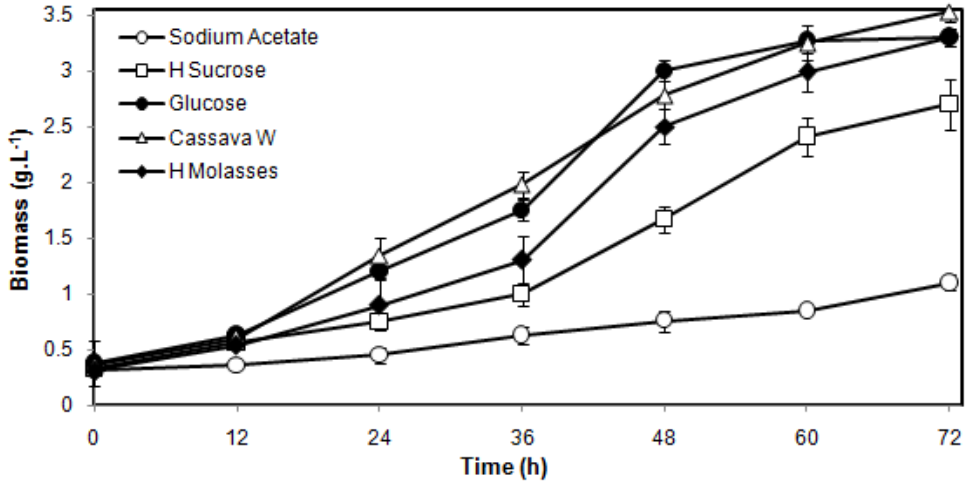

Figure 2: *Growth of C. vulgaris in different substrates in microwell plates. 'O' Sodium acetate 10 g.L^{-1}; '□' hydrolyzed sucrose 20 g.L^{-1} (9.2 ± 0.3 g.L^{-1} of glucose); '●' glucose 10 g.L^{-1}; 'Δ' cassava wastewater (9.4 ± 0.1 g.L^{-1} of glucose) '♦' hydrolyzed molasses 30 g.L^{-1} (8.9 ± 0.1 g.L^{-1} of glucose).*

Higher concentrations of substrate were used for *C. vulgaris*, because preliminary results of substrate inhibition performed in our laboratory showed that concentrations higher than 10 g.L^{-1} of glucose can inhibit the strain growth. Inhibition of *S. bijugus growth* was observed for sugar concentration above 5 g.L^{-1}. Higher initial concentration of cells was also used to *S. bijugus*, because this strain is more sensitive to substrate inhibition.

Cultivation of *C. vulgaris* with fructose resulted in cell death, indicating that this strain was unable to consume fructose. This behavior can explain which the growth rate observed when hydrolyzed molasses, cassava wastewater and hydrolyzed sucrose were used as a carbon source were similar to those observed in the cultivation with glucose. Different from the behavior observed with *S.bijugus* since this microalga is able to consume fructose.

Using sodium acetate, *C. vulgaris* produced higher biomass concentration than *S. bijugus* reaching cell concentrations of 1 g.L^{-1} in 72 hours. Glycerol was also tested because it is derived from biodiesel production processes, is abundant and is a renawable substrate of low cost. However, neither of the two tested strains were grown in glycerol. When sucrose without hydrolyze was used, cell growth was not observed for the two strains studied

Lignocellulosic biomass is another very important carbon-neutral, renewable, and sustainable feedstock. Xylose is the principal sugar released from this feedstock (Liang, 2013) and was investigated in this work since it is a inexpensive carbon source. However, none of the two strains were able to utilize this sugar.

3.3 Miniaturized culture methodology

Although the methodology of miniaturized growth allowed the screening of different substrates much faster than other methodologies, some experimental problems must be overcome in the lab, as ease of the settling of cells, evaporation of the medium during long time experiments and interference in spectrophotometric reading (caused by water condensation on the lid). To adapt the methodology of miniature culture to heterotrophic microalgae cultivation, high speed agitation (250 rpm) was used. Furthermore, smaller volume of plate wells (reaction medium 0.5 mL) were used. The microplates were discarded each time of withdrawal of the sample (12 to 12 hours) to avoid interference in reading caused by condensation of water on cover and all crops were interrupted in 72 hours.

In order to validate the microplate method, we compared the curves of growth through the $\mu_{máx}$ values (maximum specific growth rate) of *C. vulgaris* in shake-flask and microwell plates, which were studied under the same culture conditions (Figure 3).

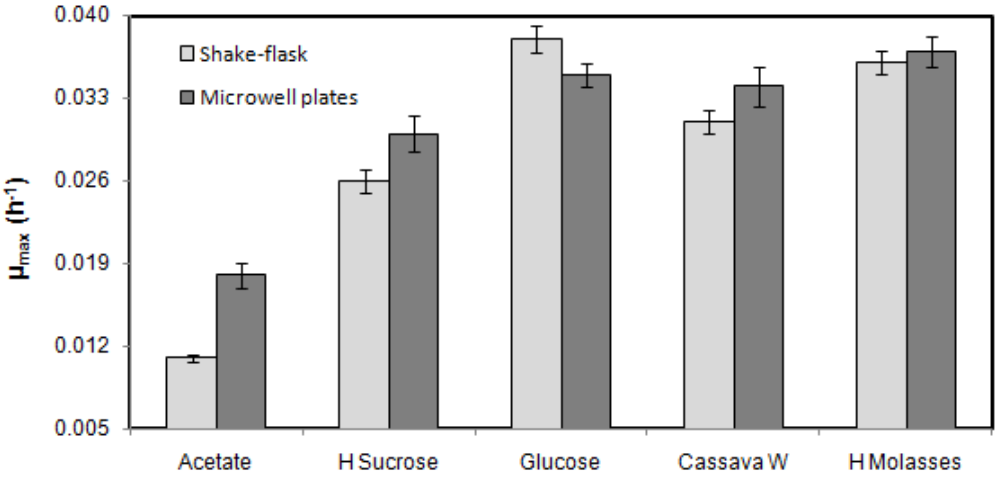

Figure 3: *Specific growth rates of C. vulgaris in different substrates tested (sodium acetate, hydrolyzed sucrose, glucose, cassava wastewater and hydrolyzed molasses) in shake-flask cultivation and microwell plates.*

By comparing the growth rates of the cultures performed in micro-plates and shake-flasks, it was observed that microplates can be very useful in process development because they can be scaled to the pilot scales. This small-scale system can be used only for a first screening, since these systems typically lack a sufficient oxygen supply (Hiliig et al. 2014), besides the other problems mentioned earlier. Normally after selecting the best strains, substrates and conditions previously used in the micro-plates, are tested in shake flasks before fermentations in larger scale bioreactor.

4. Conclusions

The results demonstrate the feasibility of the miniaturized culture methodology in the development and evaluation of heterotrophic cultivation of microalgae. The evaluated microalgae strains behaved differently according to the carbon sources tested. Cassava wastewater and sugarcane molasses hydrolyzed allowed higher biomass production and proved to be a suitable low cost substrate for increasing algae-based processes feasibility.

Acknowledgement

The authors gratefully acknowledge the support from FAPESP (Fundação de amparo à pesquisa do estado de São Paulo), CNPQ (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and Petrobras.

References

Bumbak, F., Cook, S., Zachleder, V., 2011, Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations, Appl Microbiol Biotechnol, 91, 31-46.

Canakci, M., Sanli, H., 2008, Biodiesel production from various feedstocks and their effects on the fuel properties, Journal Ind Microbiol Biotechnol, 35, 431-441.

Gao, C., Zhai, Y., Ding, Y., Wu, Q., 2010, Application of sweet sorghum for biodiesel production by heterotrophic microalga *Chlorella protothecoides*. Appl Energ, 87, 756-61.

Hiliig, F., Pilarek, M., Junne, S., Neubauer, P., 2014, Cultivation of Marine Microorganisms in Single-Use Systems, Adv Biochem Eng Biotechnol, 138, 179-206.

Huang, L., Xiang, Y., Cai, J., Jiang, L., Lv, Z., Zhang, Y., Xu, Z., 2011, Effects of three main sugars in cane molasses on the production of butyric acid with *Clostridium tyrobutyricum*, Korean Journal of Chemical Engineering, 28, 2312-2315.

Liang, Y., 2013, Producing liquid transportation fuels from heterotrophic microalgae, Applied Energy, 104, 860–868.

Malcata, F.X., 2011, Microalgae and biofuels: A promising partnership?, Trends in Biotechnology, 29, 542-549.

Mata, T.M., Antonio, A.M., Nídia S.C., 2010, Microalgae for biodiesel production and other applications: a review, Renew Sustainable Energy, 14, 217-232.

Mitra, D., Leeuwen, J.V., Lamsal, B., 2012, Heterotrophic/mixotrophic cultivation of oleaginous *Chlorella vulgaris* on industrial co-products, Algal Research, 1, 40–48

Oncel, S.S., 2013, Microalgae for a macroenergy world, Renewable and Sustainable Energy Reviews 26, 241–264.

Perez-Garcia, O., Bashan, Y., Puente, M. E., 2011, Organic carbon supplementation of sterilized municipal wastewater is essential for heterotrophic Growth and removing ammonium by the microalga C*hlorella vulgaris*, Journal of Phycology, 47, 190-199.

Perez-Garcia, O., Escalante, F.M.E., de-Bashan, L.E., Basahn, Y., 2010, Heterotrophic cultures of microalgae: Metabolism and potential products, Water Research, 45, 11-36.

Richmond, A. 2004, Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Sciences Ltd.

Satyawali, Y., Balakrishnan, M., 2008, Wastewater treatment in molasses-based alcohol distilleries for COD and color removal: A review, J Environ Manage, 86, 481-497.

Stein-Taylor, J.R., 1973, Handbook of Phycological Methods: Culture Methods and Growth Measurements, University Press.

Suali, E., Sabartly, R., 2012, Conversion of microalgae to biofuel, Renewable and Sustainable Energy Reviews, 16, 4316-4342.

Suman, P.A., Urbano, L.H., Leonel, M., Mischan, M.M., 2011, Effect of fermentation parameters on ethanol production from cassava liquid residue (manipueira), Acta Scientiarum Technology, Maringá, 33, 379-384.

Valduga, E., Valério, A., Treichel, H., Luccio, M.D., Jacques, R.A., Fúrigo A. J., 2007, Pre-treatment of molasses cane sugar and corn steep liquor for the bioproduction of carotenoids, Quimica Nova, 30, 1860-1866.

Xu, H., Miao, X., Wu, Q., 2006, High quality biodiesel production from a microalga *Chlorella protothecoides* by heterotrophic growth in fermenters, Journal of Biotechnology, 126, 499-507.

Wu, L.F, Chen, P.C., Huang, A.P., Lee, C.M., 2012, The feasibility of biodiesel production by microalgae using industrial wastewater, Bioresource Technology, 113, 14–18.

Yan, D., Lu, Y., Chen, Y-F., Wu, Q., 2011, Waste molasses alone displaces glucose-based medium for microalgal fermentation towards cost-saving biodiesel production, Bioresour Technol, 102, 6487-6493.